PATENT

Attorney Docket No.: 36290-0415-00-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent application of Patrick Gerard Johnston *et al.*	Conf. No. 1280
Serial No.:	10/580,748	Group Art Unit: 1635
Filed:	August 11, 2006	Examiner: Richard Schnizer
For:	**CANCER TREATMENT**	

DECLARATION OF DANIEL LONGLEY UNDER 37 C.F.R. 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, DANIEL LONGLEY, hereby declare and state as follows:

1. I have worked in the field of cancer studies for 10 years. I am a lecturer of the School of Medicine, Dentistry and Biomedical Sciences at the Queen's University of Belfast, a position I have held for 4 years, and I am an inventor of the present application. The Queen's University of Belfast is the Assignee of the present invention). The following experimental work was carried out by me or under my supervision.

CERTIFICATE OF MAILING
UNDER 37 C.F.R. 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date indicated below, with sufficient postage, as first class mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

BY Jennifer R. Hanna

DATE: 11/23/09

2. Figures 1 and 2 as provided in the attached Exhibit A pages 1 and 2 show the effect and efficacy of siRNA sequences SEQ ID No1 and SEQ ID No2.

3. In the studies described herein all cells were maintained in 5% CO_2 at 37°C. HCT116p53+/+ and HCT116p53-/- isogenic human colorectal cancer cells were kindly provided by Professor Bert Vogelstein (John Hopkins University, Baltimore, MD). Both HCT116 cell lines were grown in McCoy's 5A medium (Life Technologies Inc.) supplemented with 10% dialysed foetal calf serum, 50µg/ml penicillin-streptomycin, 2mM L-glutamine and 1mM sodium pyruvate. H460 cells were maintained in DMEM supplemented with 10% bovine calf serum, 1mmol/L sodium pyruvate, 2mmol/L L-glutamine and 50µg/mL penicillin/streptomycin (all from Invitrogen, Paisley, UK). A549 cells were maintained in DMEM supplemented with 10% bovine calf serum, 2mmol/L L-glutamine and 50µg/mL penicillin/streptomycin. 34LU cells were maintained in DMEM supplemented with 10% bovine calf serum, 2mmol/L L-glutamine, 100µmol/L non-essential amino acids and 50µg/mL penicillin/streptomycin. BEAS-2B cells were maintained in F12 Ham Medium (Invitrogen) supplemented with 10% bovine calf serum, 2mmol/L L-glutamine, 100µmol/L non-essential amino acids (Invitrogen) and 50µg/mL penicillin/streptomycin.

4. In the studies described herein siRNAs were designed to down-regulate either both c-FLIP splice variants (referred to as FT or FT#1), or to specifically target the long form (FL) or the short form (FS). The additional c-FLIP-targeted sequences analysed were: FT#2, AAG ATA AGC AAG GAG AAG AGT; and FT#3, AAC TGC TCT ACA GAG TGA GGC. The non-silencing control (SC) siRNA is: AAT TCT CCG AAC GTG TCA CGT . siRNAs for *in vitro* transfection were obtained from Dharmacon (Lafayette, CO). Modified (Stealth) siRNAs based on the FT (SEQ ID No1), FL (SEQ ID No2) and SC sequences were obtained from Invitrogen, Life Technologies. siRNA transfections were performed using the oligofectamine transfection reagent, according to the manufacturer's instructions (Invitrogen).

5. Western blot studies performed herein used Caspase 8 (Alexis, Bingham UK), PARP (eBioscience, San Diego) and c-FLIP (NF-6, Alexis) mouse monoclonal antibodies in conjunction with a horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody (Amersham, Little Chalfont, Buckinghamshire, England). Caspase 3 (Cell Signaling Technology) rabbit polycolonal antibody was used in conjunction with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody(Amersham). Equal Equal loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma, Poole, Dorset, UK).

6. **Figure 1 (A)** provides a Western blot showing c-FLIPL, c-FLIPS, PARP cleavage, procaspase-8 and p41/43 caspase-8 expression in HCT116p53+/+ cells 24 hours post transfection with mock (0) or 0.01, 0.05, 0.1, 0.5 and 1nM c-FLIP targeted (FT) siRNA (SEQ ID No1). Equal loading was confirmed by analysing β-Tubulin expression. **Figure 1 (B)** shows densitometric analysis of c-FLIP knockdown in the Western blot depicted in panel (A); as was assessed using a Biorad Chemidoc Molecular Imager.

7. The results shown by the Western blot demonstrate the highly potent activity of FLIP siRNA ('FT') SEQ ID No1: AAG CAG TCT GTT CAA GGA GCA. This siRNA (SEQ ID No1) is able to achieve ~75% target knockdown and induce apoptosis (as indicated by PARP cleavage and caspase 8 processing) at a concentration of 0.1nM. At 1nM of SEQ ID NO1, greater than 96% of target knockdown is achieved.

8. Siegmund et al. (Mol Med. 2002 Nov;8(11):725-32.), as cited by the examiner, is only able to achieve this level of knockdown by transfecting cells twice at concentrations of either 150nM (FLIP(S)) or 300nM (FLIP(L)).

9. Thus, the siRNA provided by SEQ ID No1 is more effective than either 150nM (FLIP(S)) or 300nM (FLIP(L)) as taught by Siegmund.

10. **Figure 2** provides a Western blot showing PARP cleavage, c-FLIPL and c-FLIPS expression in HCT116p53+/+ cells **(A)** and HCT116p53-/- cells **(B)** transfected with 1nM non-silencing control (SC), 1nM c-FLIP targeted (FT) (SEQ ID No1) or 1nM c-FLIPL specific (FL) siRNA (SEQ ID No2). Cells were co-treated with either no drug (CON), 5ng/mL rTRAIL or 100ng/mL CH-11 for 24 hours. Equal loading was confirmed by analysing β-Tubulin expression. **Figure 2 (C)** shows densitometric analysis of c-FLIP knockdown in the Western blot depicted in panel (A), lanes 1, 2 and 3. This was assessed using a Biorad Chemidoc Molecular Imager.

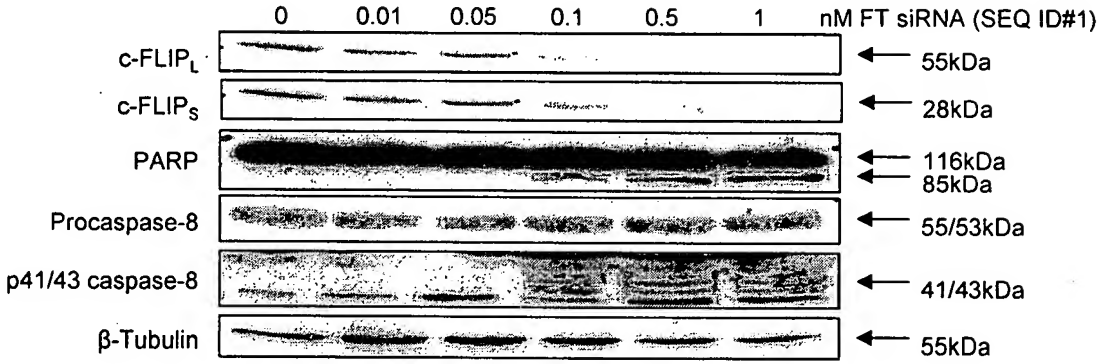
11. The results of the Western blot demonstrate that FLIP siRNA sequences FT (SEQ ID No1) and FL (SEQ ID No 2; AAG GAA CAG CTT GGC GCT CAA) are highly potent. At 1nM, FL siRNA (SEQ ID No2) more potently down-regulates c-FLIPL expression than FT siRNA (SEQ ID No1). Note that the SEQ ID No2 siRNA does not target the short splice form of c-FLIP. Both SEQ ID No1 and SEQ ID No2 siRNAs induce similar levels of apoptosis (as assessed by PARP cleavage) when administered alone, or in combination with death ligands CH-11 and TRAIL.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

20/11/09
Date

Dan Longley
Daniel Longley

A



B

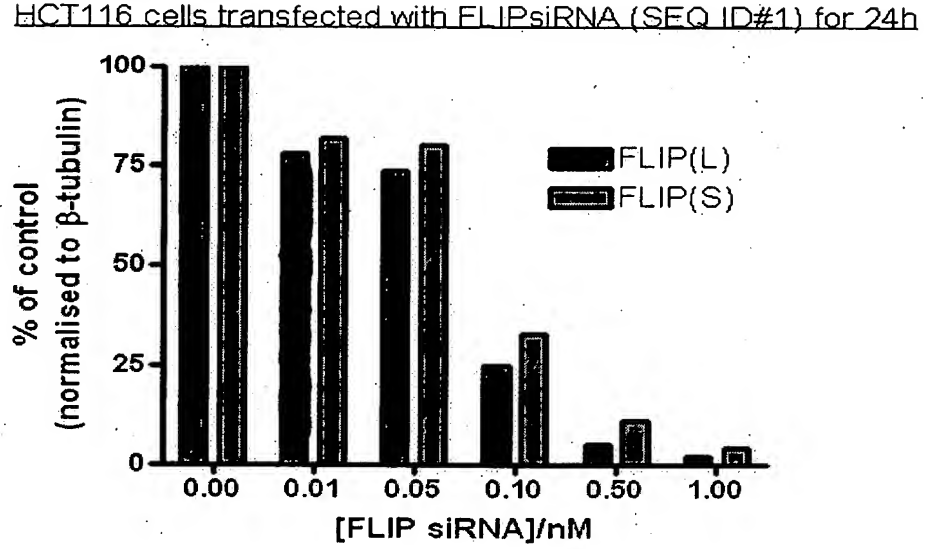
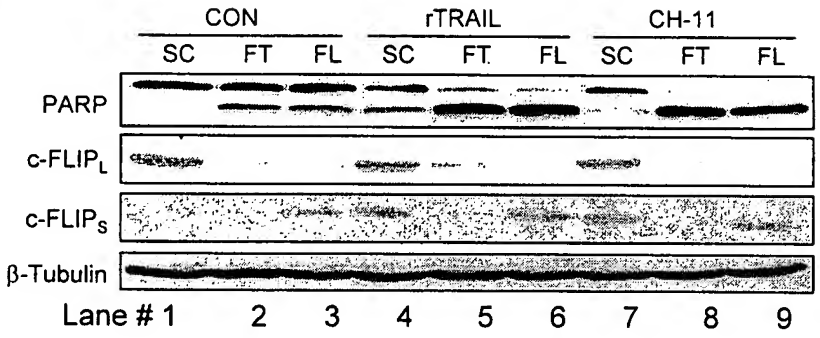


Figure 1

Exhibit A – Page 1

A



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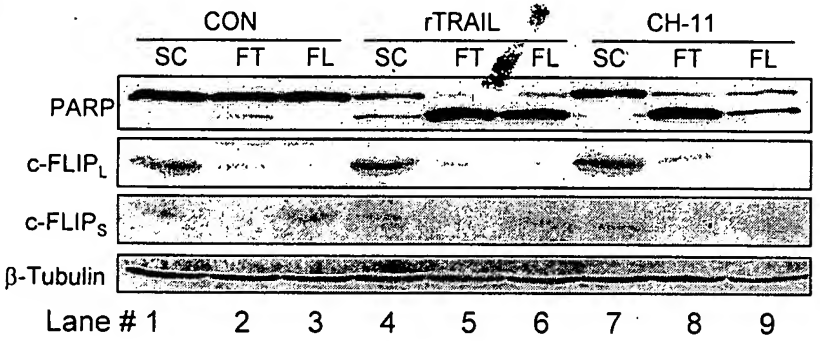

FT = SEQ ID NO1

FL = SEQ ID NO2

C

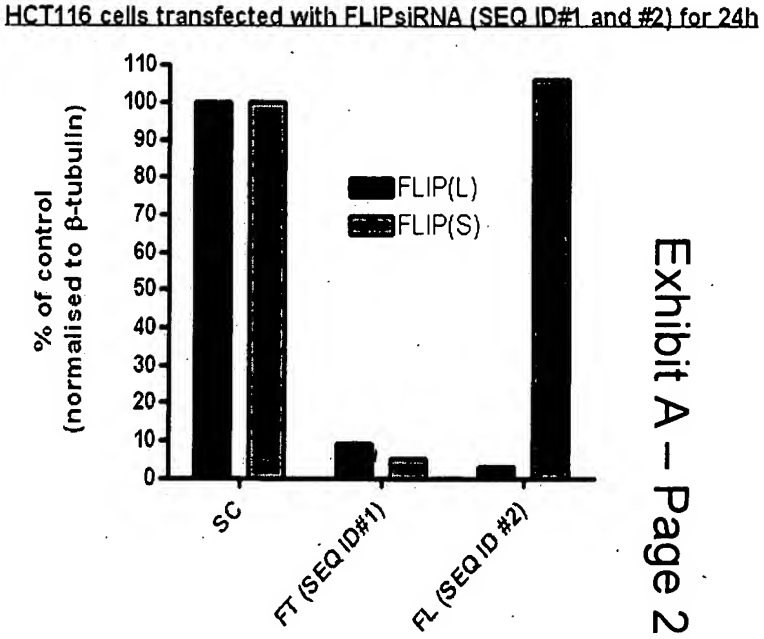

Figure 2

Exhibit A – Page 2